IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Atty. Dkt. No.: 76333/101/UNOF

In re patent application of
Joel S. GREENBERGER
Serial No.: 08/136,079
Filed: October 15, 1993
For: PROTECTION FROM IONIZING IRRADIATION OR CHEMOTHERAPEUTIC DRUG DAMAGE BY *IN VIVO* GENE THERAPY

Group Art Unit: 1804
Examiner: M. Newell

DECLARATION OF JOEL S. GREENBERGER
UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Joel S. Greenberger, declare and say as follows:

1. I am an expert in the field of experimental hematology and have 15 years experience in radiation biology and the developmental biology of gene transfer in bone marrow stromal cells and hematopoietic stem cells. I am a clinical radiation oncologist with 20 years experience in managing the pulmonary side effects of radiation therapy. Currently, I am a Professor of Radiation Oncology at the University of Pittsburgh School of Medicine and the Chairman of the Radiation Oncology Department of the Pittsburgh Cancer Institute of the University of Pittsburgh in Pittsburgh, Pennsylvania. I received my M.D. from Harvard Medical School in Boston, Massachusetts, and have authored or co-authored over 30 publications in the field of radiation biology and clinical radiation oncology. A copy of my *Curriculum vitae* is attached as Appendix I.

2. I have read and believe that I have understood the specification and claims of the above-identified patent application, Serial No. 08/136,079 ("the application").

3. I understand that the examiner has rejected the claims of the application because he alleges that the specification fails to adequately teach how to make and use the invention (i.e., fails to provide an enabling disclosure). More specifically, the examiner alleges that "[t]he field of the invention, *in vivo* gene therapy to confer normal tissue protection from treatment-related toxicities, is an emergent technology that is both unpredictable and not well-established. . . . It would not be possible for one of ordinary skill in the art to use this invention as described in the specification to provide therapeutic levels of radioprotection or chemotherapeutic protection without undue experimentation."

4. I have exciting data and information that I and members of my laboratory obtained and submitted as part of a grant application in Lung Cancer to the Public Health Service of the Department of Health and Human Services. Recent *in vivo* data from my laboratory shows that the delivery of the MnSOD transgene into the lung objectively decreases two parameters of radiation damage to the lung. Specifically, serum levels of TGFβ and pulmonary levels of both TGFβ and IL-1 do not increase to the same extent as controls that have not received the transgene. In addition, mice that have been given full lung irradiation after MnSOD transgene therapy show improved survival.

5. To determine (1) if MnSOD expression was detectable after MnSOD transgene introduction and (2) if there was a detectable reduction in irradiation-induced pulmonary toxicity as a result of such MnSOD transgene expression, we compared our MnSOD plasmid containing the CMV promoter to pRK5 alone, β-gal alone, or β-gal mixed with our MnSOD plasmid, each using the DC-chol liposome delivery system. Gao, X. and Huang, L., *Biochem. Biophys. Res. Comm.* 179(1): 281-285 (1991). Thereafter, groups of mice received 1500 cGY, 2000 cGY, 2500 cGY or 3000 cGY hemibody irradiation. MnSOD transgene-specific mRNA

was detected by reverse transcriptase-polymerase chain reaction (RT-PCR) in lung fragments removed from injected mice at 2 days after direct intratracheal injection of the MnSOD plasmid construct, was measured.

6. The results of the experiments described in paragraph 4 demonstrated that the MnSOD transgene was detectable in lungs of 1500 cGY irradiated mice using RT-PCR and primers specific for the human MnSOD transgene, compared to irradiated mice or unirradiated non-injected mice. Also, RNA for TGF-β, IL-6, IFN-α and TNF-α was measured at 1, 3, 6, 24, 48 and 72 hours after 2000 cGY irradiation in lungs of control irradiated, compared to MnSOD transgene, intratracheally-injected mice. Prior studies showing RNA levels for cytokine transcripts in irradiated lung tissue did not look at these early time points. The data in Figures 1-3 show levels of mRNA for IL-1 and TGF-β at 6 hours, 24 hours and 48 hours after 2000 cGY, and the protective effect of the MnSOD transgene delivered intratracheally. A dose-response study of the effect of irradiation on TGF-β mRNA is shown in Table 1. After 72 hours, it was clear that irradiation effectively decreased detectable levels of TGF-β mRNA, as shown in Figures 1-2, and IL-1 mRNA, as shown in Figure 3. Table 1 shows serum TGF-β levels which rise after total body irradiation, but are less elevated in animals that have received MnSOD (pRK-5 plasmid liposomes) transgene therapy.

7. Lung fragments removed at 7 days after 1500 cGY irradiation of MnSOD-pRK5-liposome-treated mice (in the experiment described in Table 1) demonstrated less acute changes than those observed in control mice. Although these histopathology results are preliminary, the biochemical and molecular biologic data are clearly suggestive of biochemical (TGF-β) and molecular biologic (IL-1, TGF-β by RT-PCR) evidence of a protective effect of the human MnSOD transgene expression

delivered by intratracheal injection 24 hours prior to irradiation of mouse lungs *in vivo*. In another large experiment shown in Figure 5, we have clearly detected increased survival out to 120 days of MnSOD-transgene-treated mice receiving 2000 cGY or 3000 cGY to both lungs [Figures 5A and 5B, respectively]. Please note that the figures only indicate increased survival out to 60 days; however, the longer 120 day survival was measured after the figure was prepared.

8. We have developed an orthotopic model of human primary lung cancer using subcarinal injection of 1 X 10^6 Lewis lung carcinoma cells. Mice with orthotopic tumors were screened by diagnostic x-ray, as shown in Figure 6, and entered in gene therapy/radiotherapy experiments when localized tumors reach 0.3-0.4 cm in diameter, as shown in Figure 6A. Mice with tumors were intratracheally injected with 20 μg of liposomes in 50 μl (concentration of 400 μg/ml) of pRK5-MnSOD-plasmid-liposomes, or 500 μg of β-gal plasmid/liposomes. Hemibody irradiation was delivered the next day to 10 mice per group receiving 500, 1000, 1500, 2000, 2500 or 3000 cGy with or without midline esophageal block at 100 cGy/minute. One animal in each group was sacrificed at days 1, 3, 7, 10 or 14 after irradiation. In each sacrificed animal, the following parameters were measured: (a) tumor size; (b) tumor and normal lung tested for transcription of the human MnSOD transgene by RT-PCR; and the transcription levels of RNA for TGF-β, TNF-α, IL-6 and IFNγ were detected. The data showed significant decrease in tumor size in a direct dose response fashion. Lung toxicity was decreased in mice that received MnSOD-plasmid-liposomes, as measured by pathologic changes and stable mRNA levels for Il-1, TGF-β and the other inflammatory cytokines tested. In contrast, non-treated mice showed a significant increase in detectable mRNA for these cytokines at 24 hours. The levels of TGF-β at three days, one week, two weeks and one month after MnSOD inhalation transgene therapy prior to irradiation were also decreased compared to tumor-bearing,

irradiated, but nontransgene-treated mice. Table 4 illustrates the results of gene therapy prior to irradiation decreases lung toxicity, with similar tumor shrinkage. The decreased lung toxicity, illustrated in Figure 7, has likely lead to the encouraging preliminary data showing improved survival.

9. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this patent.

Respectfully submitted,

11/14/95
Date

Joel S. Greenberger

FIG 1: TGF-ß mRNA Expression Normalized to G3PD in Lungs of MnSOD-Plasmid-Liposome-Treated Mice

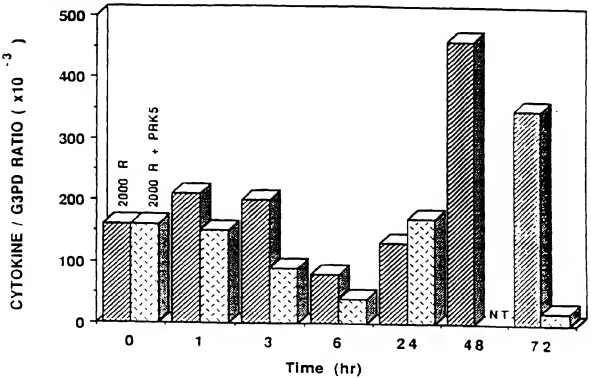


FIG 2: TGF-ß mRNA Expression Normalized to Actin in Lungs of MnSOD-Plasmid-Liposome-treated Mice

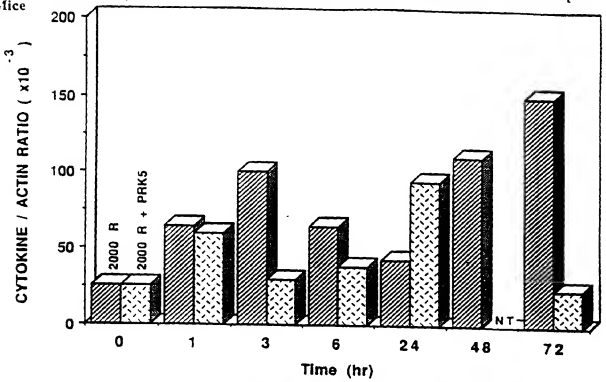
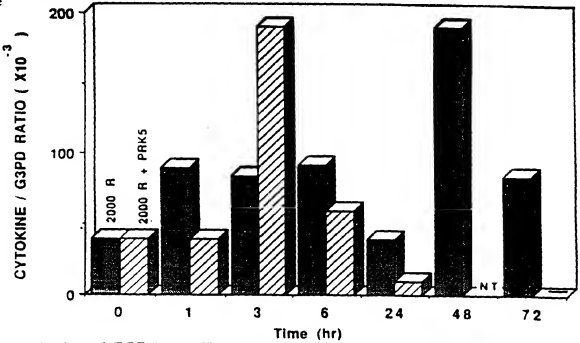


FIG 3: IL-1 mRNA Expression Normalized to G3PD in Lungs of MnSOD-Plasmid-Liposome-Treated Mice



FIGS. 1-3: Quantitation of TGF-ß and IL-1 transcript induction by ratio of P32 counts normalized to actin or G3PD. Levels of RT-PCR in lungs removed from 2000 cGy total-lung-irradiated or MnSOD-plasmid/liposome-treated mice. Mice were irradiated on day 0, intratracheal liposomes were delivered on day (-1). A third group given LacZ plasmid/liposomes was not different from the control group [Table 3] (see Methods).

Table 1: TGF-ß mRNA expression at 7 days in irradiated lungs of mice that had esophageal protection with a midline bl ck and MnSOD delivery by liposomes (See Figs. 11-12)

	Irradiation D se (cGy)			
Treatment[1]	**1500**	**2000**	**2500**	**3000**
Hemibody Irradiation Alone	320	5666	136	7778
Midline Block (MB) and Irradiation Alone	199*	2810*	2100*	585*
pRK5-MnSOD	3705	965‡	225‡	0‡
pRK5-MnSOD (MB)	222	7271	2210	358‡
LacZ	2978	1802	2220	NT

1. C57BL/6J mice were divided into three groups, one was injected intratracheally with pRK5-MnSOD plasmid-liposomes (DC-chol [98]) [Fig. 10], one with plasmid containing the LacZ gene and one with no gene therapy. The mice were then irradiated 24 hours later to 1500 cGy, 2000 cGy, 2500 cGy or 3000 cGy. Subgroups were protected by a midline esophageal block (See Figs. 11-12). Seven days later, the lungs were removed and the RNA isolated. RT-PCR was used to amplify the mRNA for TGF-ß1 which was transferred to nitrocellulose membranes and probed for TGF-ß1 mRNA. After exposure to the membranes, x-ray film was developed and analyzed by spectrophotometry with the results shown about in densitometric units. * Significant decrease compared to hemibody irradiation alone. ‡Significant decrease compared to non-pRK5-treated mice. (NT = not tested).

Table 2: Serum TGF-ß1 levels at various times following MnSOD-plasmid-liposomes and total lung irradiation of C57BL/6J mice (effect of fractionation of x-ray dose)

Time[1] (hour)	Irradiation Control	pRK5-MnSOD	LacZ Alone
0	0	0	0
1	754	0	0
3	396	0	NT
6	626	0‡	679
24	256	0‡	368
48	428	NT	NT
72	113	0‡	NT

1. C57BL/6J mice were divided into three groups: one group received an intratracheal injection of pRK5-MnSOD-plasmid-liposomes [Fig. 10], one received LacZ plasmid-liposomes and the third group received no injection (control). The mice were then irradiated 24 hours later to both lungs using a midline block (MB) [Figs. 11-12] by (fractionated irradiation) 500 cGy/day for four consecutive days. AT 0, 1, 3, 6, 24, 48 or 72 hours following the final irradiation treatment (Time 0 = day 5 after pRK5-MnSOD), blood was collected and serum TGF-ß1 levels were quantitated using a TGF-ß1 ELISA kit (R&D Systems, Minneapolis, MN). Results are presented as pg/100 µl of serum. (NT = not tested). ‡Significant difference compared to irradiated control.

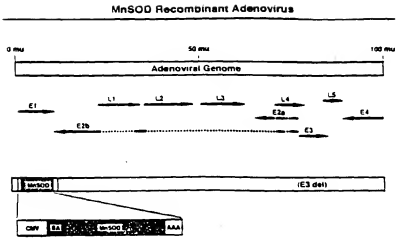


FIG. 4: **Construct used in liposome and adenovirus-MnSOD lung radiation protection experiments. MnSOD is a human transgene. RT-PCR primers were designed to detect human MnSOD adenovirus sequences, not endogenous mouse MnSOD (3A).**

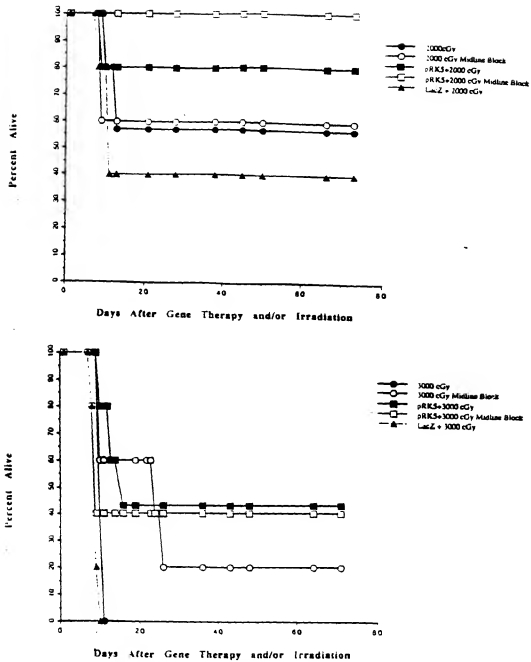
Table 3: Effect of intratracheal pRK5-MnSOD-plasmid-liposome therapy on the 7 day d se-response induction of serum TGF-ß1 levels in total lung irradiated mice

Treatment Group	1500 cGy	2500 cGy
Hemibody Irradiation Alone	1319	894
Hemibody Irradiation Plus Midline Block (MB) Alone	736*	489*
pRK5-MnSOD	110‡	506‡
pRK5-MnSOD-(MB)	483*	285*
LacZ-(MB)	959	872

1. C57BL/6J mice were divided into three groups: one received an intratracheal injection of 500 µg of pRK5-MnSOD plasmid-liposomes (DC-chol [98] and [Fig. 10]), one received an intratracheal injection of plasmid containing a LacZ gene, and a third group received no gene therapy. The mice were irradiated 24 hours later to 1500 cGy or 2500 cGy. Seven days later, serum was collected and TGF-ß1 levels were determined using a TGF-ß1 ELISA kit (R&D Systems, Minneapolis, MN). Results are presented as pg/100 µl of serum. MB = midline block for esophagus protection, as described in the Methods.

*Significant decrease compared to irradiation without midline block.

‡Decrease compared to hemibody irradiation without MnSOD gene therapy.

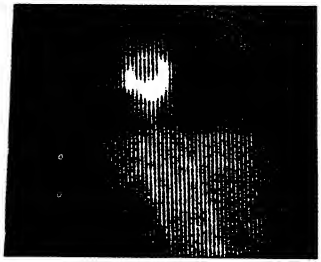


B

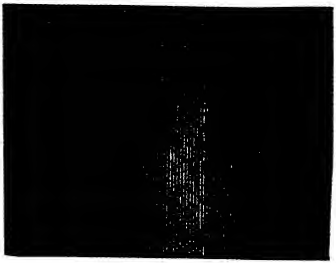
FIGS. 5A-5B: Survival advantage of plasmid-liposome [DC-chol _] MnSOD-treated mice after hemibody irradiation. C57BL/6J mice were divided into 12 groups. Six groups received 2000 cGy (A), and six groups received 3000 cGy (B) to a hemibody field including the entire pleural cavity. pRK5-MnSOD plasmid/liposomes or LacZ liposomes were injected intratracheally into designated groups of mice 24 hours before irradiation [20 μg of liposomes in 50 μl (concentration of 400 μg/ml)]. Mice were shielded, so that only the lungs were irradiated, and groups receiving midline block were placed under a device which also shields the esophagus and spinal cord The groups were treated as follows: irradiation alone, ( ); irradiation plus esophageal protection, midline block alone, (O); pRK5-MnSOD, (■); pRK5-MnSOD plus midline block, (□); or LacZ gene, (▲); Conclusion: In the 2000 cGy groups, pRK5-MnSOD groups survived longer than irradiated control or LacZ-treated mice. In the 3000 cGy groups, midline block and pRK5-MnSOD or pRK5-MnSOD groups survived longer (40% alive at 60 days) than all other groups.

ig. 6: Effect of MnSOD plasmid-liposome gene therapy on irradiated 500 cGy x4 mic with orthotopic Lewis lung carcinoma

6 (A) - 7 Days
ontrol Tumor-Bearing Not Irradiated
ote tumor in right hemithorax)



6 (B) - 7 Days
Tumor-Bearing MnSOD-Plasmid/Liposome, Irradiated
(note smaller volume of tumor in right hemithorax)



6(C) - 7 Days
Tumor-Bearing B-Gal Plasmid/Liposome, Irradiated
(note small tumor volume in right hemithorax)

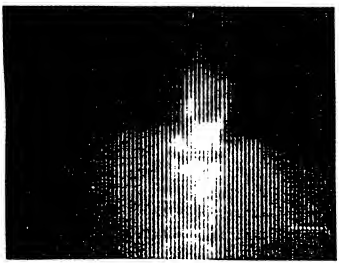


TABLE 4: Pathol gic evaluation of MnSOD plasmid/liposome-treated/irradiated mice with orthotopic, subcarinal Lewis lung tum rs

Group of C57BL/6J mice (Injected with) orthotopic Lewis lung carcinoma (0.5 cm tumor mass at carina)	MnSOD transgene detected in lung/tumor. n= number of mice tested[2]	Lung Pathology[1] (respectively for each mouse in respective column)
Control irradiated (2000 cGy)	n=3 (0,0,0)/(0,0,0)	3+, 3+, 3+
MnSOD-plasmid/liposome	n=3 (+,+,+/0,0,0)	2+, 1+, 3+
ß-gal-plasmid/liposome	n=4 (0,0,0,0/0,0,0,0)	3+, 3+, 3+, 3+
MnSOD liposomes plus tumor (plus 2000 cGy)	n=3 (+,+,+/0,0,0,0)	2+, 3+

1. Lung sections were scored for evidence of edema, exudate and cellular infiltrate as index of irradiation damage (3+, all criteria), 2+, 2 of 3; 1+ only edema seen). Data at 7 days after irradiation, 8 days after gene therapy (20 days after orthotopic tumor injection).

2. Normal lung and tumor removed for RT-PCR detection of MnSOD transgene as described in the Methods.

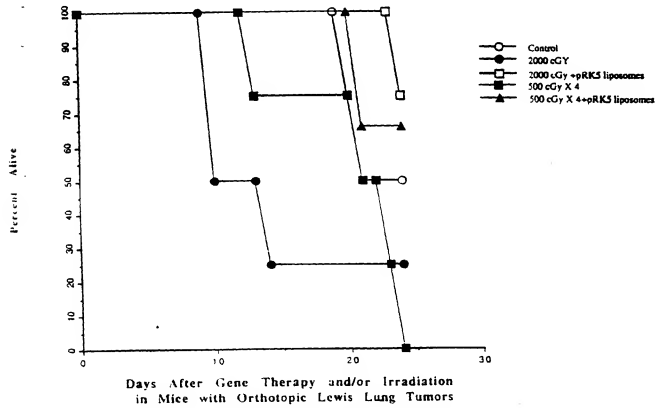


FIG. 7: Gene therapy plasmid-liposomes (DC-chol) improves total lung irradiation tolerance of mice with 0.3-0.4 cm orthotopic Lewis lung tumors. Mice were treated and evaluated as described in the legend to Figs. 5A-6A, except that these had 0.3-0.4 cm orthotopic tumors [Fig. 6A]. Gene therapy by intratracheal injection was on day zero. Irradiation was delivered on day one for single dose group 200 cGy. The fractionated dose groups received treatments on days one, two, three and four. Conclusion: Group 2000 cGy plus pRK5 plasmid-liposomes (□) survived longer than either irradiation group.

Joel S. Greenberger Professor and Chairman

EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Columbia Univ. New York, NY	B.A.	1967	Pre-Medicine
Harvard Medical School, Boston, MA	M.D.	1971	Medicine
Boston City Hospital, Boston, MA	Intern	1972	Medicine
Joint Center for Radiation Therapy, Harvard Boston, MA	Resident	1974-1977	Radiation Oncology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. DO NOT EXCEED TWO PAGES.

1974 Research Assoc., Lab. of RNA Tumor Viruses, NIH, Bethesda, MD.
1977 Research Assoc., Dept. of Microbiology, Harvard Medical School.
1979 Assistant Professor of Radiation Therapy, Harvard Medical School.
1982 Associate Professor of Radiation Therapy, Harvard Medical School.
1984 Professor and Chairman, Dept. Radiation Oncology, Univ. Mass. Med. Sch.
1993 Professor and Chairman, Dept. Radiation Oncology, Univ. Pitt. Med. Sch./Deputy Director of the Pittsburgh Cancer Institute.

HONORS:

Thesis, Harvard Medical School, "Two Approaches To Quantitation Of Leukemic Leukocyte Functions In Clinical Disease" Cum Laude, 1971.
Sara Stone Burns Award of the American Cancer Society, Mass. Div., for innovative research grant proposal.

PUBLICATIONS: (Representative pertinent list from last seven years from list of 201 publications)

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2. Greenberger JS, Anklesaria P. Recent studies of the hematopoietic microenvironment in long-term bone marrow cultures. Immunologic Res, 8:226-235, 1989.
3. Ohta M, Anklesaria P, Wheaton M, Ohara A, Pierce JH, Holland CA, Greenberger JS. Retroviral src gene expression in continuous marrow culture increases the self-renewal capacity of multilineage hematopoietic stem cells. Leukemia, 3:206-226, 1989.
4. FitzGerald TJ, Henault S, Santucci MA, Anklesaria P, Zak S, Kase K, Farber N, Greenberger JS. Increase in low-dose-rate x-irradiation resistance of factor dependent hematopoietic progenitor cells by recombinant murine GM-CSF. Int J Rad Oncol, Biol & Phys, 17:323-335, 1989.
5. Enver T, Nakamoto B, Karlinsey J, Josephson B, Greenberger J, Papayannopoulou T. Erythropoietin changes the globin program of an interleukin 3-dependent multipotential cell line. PNAS, USA, 85:9091-9095, 1988.
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7. Anklesaria P, FitzGerald TJ, Kase K, Ohara A, Bentley S, Greenberger JS. Improved hematopoiesis in anemic Sl/Sld mice by therapeutic transplantation of a hematopoietic microenvironment. Blood, 73:1144-1152, 1989.
8. Matsui T, Pierce JH, Fleming TP, Greenberger JS, LaRochelle WJ, Ruggiero M, Aaronson SA. Independent expression of human α or β PDGF receptor cDNAs in a naive hematopoietic cell leads to functional coupling with mitogenic and chemotactic signaling pathways. PNAS, USA, 86:8314-8319, 1989.

9. FitzGerald TJ, Henault S, Sakakeeny MA, Santucci MA, Pierce JH, Anklesaria P, Kase K, Lovington K, Greenberger JS. Resistance of hematopoietic stem cells to x-irradiation at clinical low-dose-rate is induced by several classes of oncogenes. Rad Res, 122:44-53, 1990.
10. Greenberger JS, Wright E, Henault S, Sakakeeny MA, Anklesaria P, FitzGerald TJ, Wheaton M, Kase K, Kuzawa D. Hematopoietic stem cell and marrow stromal cell specific requirements for gamma-irradiation leukemogenesis in vitro. Exp Hematol, 18:408-416, 1990.
11. Santucci MA, FitzGerald TJ, Harigaya K, Woda B, Sakakeeny MA, Anklesaria P, Kase K, Holland CA, Greenberger JS. Gamma-irradiation response of cocultivated bone marrow stromal cell lines of differing intrinsic radiosensitivity. Int J Rad Oncol Biol & Phys, 18:1083-1092, 1990.
12. Anklesaria P, Teixido J, Laiho M, Pierce J, Greenberger JS, Massague J. Cell homing and proliferation mediated by membrane TGF-α binding to EGF receptor. PNAS, USA, 87:3289-3293, 1990.
13. Anklesaria P, Greenberger JS, FitzGerald TJ, Wicha M, Campbell A. Reduced expression of haemonectin may contribute to the defective hematopoiesis of Steel mutant mice. Blood, 77:1691-1698, 1991.
14. Miyake K, Weissman IL, Greenberger JS, Kincade PW. Evidence for a role of the integrin VLA-4 and a possible ligand in lympho-hemopoiesis. J Exp Med, 173:599-607, 1991.
15. Greenberger JS, Leif J, Crawford D, Anklesaria P, English D, Sakakeeny MA, Rubin JR, Pierce PH, Shadduck RK, FitzGerald TJ. Humoral and cell surface interactions during gamma-irradiation leukemogenesis in vitro. Exp Hematol, 20:92-102, 1992.
16. Greenberger JS, Sakakeeny M, Leif J, Anklesaria P, Pierce JH, Shadduck RK. Expression of M-CSF and its receptor (c-fms) by factor dependent hematopoietic progenitor cell lines evolving from cocultivation with gamma irradiated marrow stromal cell lines. Leukemia, 6:626-633, 1992.
17. Teixido J, Hemler ME, Greenberger JS, Anklesaria P. Role of β1 and β2-integrins in the adhesion of human CD34+ hi stem cells to bone marrow stroma. J Clin Invest, 90:358-367, 1992.
18. Gimble J, Youkhana K, Hua X, Wang C-S, Bass H, Medina K, Greenberger JS. Adipogenesis in a myeloid supporting bone marrow stromal cell line. J. Cell Biochem, 50:73-82, 1992.
19. Ohara A, Anklesaria P, Schultz L, Greenberger JS. Biological characterization of permanent clonal bone marrow stromal cell lines derived from "moth-eaten" Me^v/Me^v and SCID mice. J. Cell Cloning., 10:(1):33-47, 1992.
20. Neben S, Anklesaria P, Greenberger JS, Mauch P. Quantitation of murine hematopoietic stem cells in vitro by limiting dilution analysis of cobblestone area formation on a clonal stromal cell line. Exp Hematol, 21:438-444, 1993.
21. Santucci MA, Anklesaria P, Laneuville P, Das IJ, Sakakeeny MA, FitzGerald TJ, Greenberger JS. Expression of p210 bcr/abl increases hematopoietic progenitor cell radiosensitivity. Int J Rad Oncol Biol & Phys, 26:831-836, 1993.
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23. Liggett W, JRr., Shevde N, Anklesaria P, Sohoni S, Greenberger J, Glowacki J. Effects of macrophage colony stimulating factor and granulocyte-macrophage colony stimulating factor on osteoclastic differentiation of hematopoietic progenitor cells. Stem Cells, 11:398-411, 1993.
24. Shevde N, Anklesaria P, Greenberger JS, Bleiberg I, Glowacki J. Evidence for director or stromal cell-mediated stimulation of osteoclastogenesis. Proc Soc Exp Biol Med, 205:306-316, 1994.
25. Liggett WH, Jr., Lian JB, Greenberger JS, Glowacki J. Osteocalcin promotes differentiation of putative osteoclast progenitors from murine long-term bone marrow cultures. J Cell Biochem, 56:190-199, 1994.
26. Santucci MA, Pierce JH, Anklesaria P, Fortuna A, Frezza G, Babini L, Greenberger JS. Erythropoietin acts as a radioprotector on pure hematopoietic progenitor cells expressing the cognate receptor. Stem Cells, 12:506-513, 1994.
27. Epperly M, Santucci MA, Reed J, Shields D, Halloran A, Greenberger JS. Expression of the human BCL-1 transgene increases the radiation resistance of a hematopoietic progenitor cell line. Radiation Oncol Invest: Basic&Clinc Res., 2:77-83, 1994.
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29. Pogue-Geile KL, Sakakeeny MA, Panza JL, Sell SL, Greenberger JS. Cloning and expression of unique murine macrophage colony stimulating factor transcripts. Blood, (In Press).
30. Rosenstein M, Epperly M, Hughey R, Prezioso J, Greenberger JS. Overexpression of the gamma glutamyltranspeptidase transgene does not alter the gamma irradiation sensitivity of the IB3-1 normal bronchoepithelial or A549 human lung carcinoma cell line. Rad Oncol Invest: Clinc and Basic Res. (In Press).

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Atty. Dkt. No.: 76333/101/UNOF

In re patent application of
Joel S. GREENBERGER
Serial No. 08/136,079
Filed: October 15, 1993
For: PROTECTION FROM IONIZING IRRADIATION OR CHEMOTHERAPEUTIC DRUG DAMAGE BY *IN VIVO* GENE THERAPY

Group Art Unit: 1804
Examiner: M. Newell

DECLARATION OF MICHAEL LOTZE
UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Michael Lotze, declare and say as follows:

1. I am an expert in the field of gene therapy and have over 17 years experience in Cellular Immunology and over 8 years experience in Human Gene Therapy. Currently, I am a Professor of Molecular Genetics and Biochemistry and the Co-Director the Biological Therapeutics Program at the University of Pittsburgh Cancer Institute in Pittsburgh, Pennsylvania. I am also a Professor of Surgery and the Chief of Surgical Oncology at the University of Pittsburgh Cancer Institute. I received my M.D. from Northwestern University Medical School in 1974, and have authored or co-authored 250 publications in the fields of immunology and gene therapy. I serve on the editorial boards of 14 pier review scientific journals, including *Gene Therapy (Nature)*, *Cytokines and Molecular Therapy*, *Cancer Gene Therapy and Cancer Research, Therapy and Control*. A copy of my *Curriculum vitae* is attached as Appendix I.

2. I have read and believe that I have understood the specification and claims of the above-identified patent application, Serial No. 08/136,079 ("the application").

3. I understand that the examiner has rejected the claims of the application because he alleges that the specification fails to adequately teach how to make and use the invention (i.e., fails to provide an enabling disclosure). More specifically, the examiner has made three allegations. First, he claims that the animal data does not provide a reasonable expectation of success in humans. ("[T]he lack of working examples in the specification regarding *in vivo* transfer of therapeutically significant level of a therapeutic gene in humans or animals, one skilled in the art would not readily accept on its face *in vivo* transduction of therapeutic genes at clinically significant levels in any animal without undue experimentation and with a reasonable expectation of success. . . . One skilled in the art would not readily accept the disclosed animal data as correlative of results in human clinical applications based on the state of the art in the field.") Second, the examiner alleges that liposome-mediated gene transfer in mice provides neither a reasonable expectations of success in non-murine subjects nor a reasonable expectation of success with other transient vector systems. ("One skilled in the art would not readily accept on its face that disclosure of liposome-mediated gene transfer of a therapeutic gene in an animal model would be correlative of therapeutic gene transfer in any and all subjects using any and all transient vector systems.") Third, the examiner alleges that protection from radiation toxicity by gene delivery of manganese superoxide dismutase in mice provides no reasonable expectation of success for (a) delivery to non-murine subjects, (b) protection from other toxicities (*e.g.*, chemotherapy) and (c) gene delivery of other proteins capable of neutralizing or eliminating toxic ionic species (*e.g.*, metallothionein and gamma glutamyl transpeptidase). ("In light of the fact that the toxicities of radiotherapy and chemotherapy are mediated by different toxic agents, and the three protective enzymes of the claimed invention [*i.e.*, manganese superoxide

dismutase, m tallothionein and gamma glutamyl transpeptidase] act by different mechanisms to neutralize chemically distinct toxic agents, one skilled in the art would not readily accept on its face that protection from the toxicity of one mode of therapy (radiation) by gene delivery of one protective gene (MnSOD) in a mouse model would be correlative of protection from other distinct mode of therapy (such as chemotherapy) by gene delivery of other protective genes, which neutralize different toxic agents than does MnSOD.")

4. I have examined the exciting data and information that Dr. Greenberger and members of his laboratory obtained and submitted in Dr. Greenberger's declaration of November 14, 1995, as well as his most recent data presented at the ASTRO meeting in Miami, Florida, showing that the delivery of the MnSOD transgene into the lung objectively decreases radiation damage to the lung and increases animal survival.

5. The results of Dr. Greenberger's experiments demonstrate that serum TGF-β levels are less elevated in animals that have received MnSOD (liposomes) transgene therapy. Also, MnSOD-liposome-treated mice demonstrated less acute changes than those observed in control mice. These biochemical and molecular biologic data are clearly suggestive of biochemical (TGF-β) and molecular biologic (IL-1, TGF-β by RT-PCR) evidence of a protective effect of the human MnSOD transgene expression delivered by intratracheal injection 24 hours prior to irradiation of mouse lungs *in vivo*. Also, animals have an increased survival of at least 120 days after MnSOD-transgene-treated mice receiving 2000 cGY or 3000 cGY to both lungs. The orthotopic model of human primary lung cancer experiments shows that the MnSOD-liposomes and β-gal-liposomes have a significant decrease in tumor size in a direct dose response fashion. Lung toxicity was decreased in mice that received MnSOD-plasmid-liposomes, as measured by pathologic changes and stable mRNA

levels for Il-1, TGF-β and the other inflammatory cytokines tested. Also, gene therapy prior to irradiation decreased lung toxicity, with similar tumor shrinkage. It is likely that the decreased lung toxicity lead to the encouraging preliminary data showing improved survival.

6. Animal data in suitable murine or other models can predict success in human clinical trials. It is critical that an appropriate model be chosen, and oftentimes the requirement for successful application in humans is only demonstrated after years of clinical trials. *In vivo* transduction of therapeutic genes at clinically significant levels in the mouse, however, provides a reasonable expectation of success for a similar *in vivo* transfer of therapeutically significant level of a therapeutic gene in humans or other animals. The disclosed animal data are predictive of human clinical applications.

7. Liposome mediated gene transfer has been demonstrated to be successful in a variety of human clinical trials including delivery of the CFTR gene in cystic fibrosis and an immunogen, the allogeneic HLA-B7 gene, in clinical cancer trials. I do believe that the available clinical evidence as well as the liposome mediated gene transfer in mice provides a reasonable expectation of success in human subjects as well as a reasonable expectation of success with other transient vector systems. I readily accept that disclosure of liposome-mediated gene transfer of a therapeutic gene in the murine animal model is reasonably predictive of success for therapeutic gene transfer in other animals, including humans. Also, the use of liposome-mediated gene transfer is reasonably predictive of success using other transient vector systems.

8. I believe that delivery of manganese superoxide dismutase in mice does indeed provide a reasonable expectation

of success for delivery to humans. Again, clinical trials often take years to substantiate early studies in mice.

9. The end result of chemotherapy or radiation therapy are often very similar. These involve the delivery of toxic metabolites or alteration in DNA which also eventually leads to apoptosis or necrotic tumor death. The protective enzymes of the claimed invention manganese superoxide dismutase, metallothionein, and gamma glutamyl transpeptidase act by the same mechanism to neutralize chemically distinct toxic agents. This is not my area of expertise but I have examined the information provided by Dr. Greenberger and I believe that they are essentially correct. The toxicities of radiation therapy and chemotherapy both produce radical oxygen species in tissue. Thus, one need only show protection with irradiation to make a reasonable extension of this data logical to chemotherapy. There are numerous examples of this in stem cell toxicity. The three protective enzymes of the claimed invention, manganese superoxide dismutase, metallothionein and gamma glutamyl transpeptidase, each act by the same mechanism to neutralize chemically distinct toxic agents. Therefore, I readily believe that protection from the toxicity of one mode of therapy, such as radiation, by gene delivery of one protective gene (MnSOD) in a mouse model would provide a reasonable expectation for protection from another therapies that produce radical oxygen species, such as chemotherapy.

10. The general applicability of the approach of delivering genes such as superoxide dismutase can be readily applied to other gene products which are capable of neutralizing or eliminating toxic species. In other words, the protection from radiation toxicity by gene delivery of manganese superoxide dismutase in mice provides a reasonable expectation of success for protection using delivery of other protective genes capable

of neutralizing or eliminating toxic ionic species, such as metallothionein and gamma glutamyl transpeptidase.

11. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this patent.

Respectfully submitted,

4/-1-96
Date

Michael Lotze, M.D.

III. "Obviousn ss" Rej ctions Und r § 103

Claims 1-4, 9-12, 14-15 and 18-30 are rejected under 35 USC §103. It seems that the examiner has found claims 5-8, 13, 16 and 17 to be novel and non-obvious in view of the prior art.

There are four separate obviousness rejections: (1) claims 1, 4 and 20-27 are rejection under 35 USC §103 as being unpatentable over Sorrentino *et al.* in view of Mulligan; (2) claims 1-4, 9-12, 14, 18, 20-26 and 28-30 are rejected under §103 over Petkau in view of Alton *et al.*, Jaffe *et al.* and Wu *et al.*; (3) claims 1, 4, 9-12, 15, 19-26 and 28-30 are rejected under §103 over Lohrer *et al.* and Matsubara *et al.* in view of Mulligan as applied above; and (4) claims 1-3, 9-11, and 28-30 are rejected under §103 over Hockenberry *et al.* in view of Mulligan.

Each of the four rejections is improper because the examiner has not shown that the prior art as a whole suggests the desirability to selectively combine the references he has chosen. The examiner presents no line of reasoning as to why the artisan reviewing only the collective teachings of the references would have found it obvious to selectively pick and choose various elements from the several references relied on to arrive at the claimed invention. The examiner has done little more than cite references to show that one or more elements or some combinations thereof is known.

The claimed invention is not directed to one or more new elements *per se*; rather, Dr. Greenberger has invented a new method employing a new combination of elements. In fact, the references cited by the Examiner as disclosing particular elements of the invention do not actually disclose the elements of the claimed invention. The prior art cited within the instant specification and provided in the Information Disclosure Statement do disclose the particular elements of the claimed

invention. It is impermissible for the examiner to use the claims as a frame and the prior art references as a mosaic to piece together a facsimile of the claimed invention.

The first obviousness rejection of claims 1, 4, and 20-27 over Sorrentino in view of Mulligan admits that the Sorrentino reference is directed to *ex vivo* gene transfer, rather than *in vivo* gene therapy. But the method of Sorrentino differs from the instant invention in several other respects: (1) retroviral vectors are used, (2) stable expression is desired and achieved and (3) the encoded protein does not neutralize or eliminate the toxic ionic species, but rather, extrudes it. The Mulligan reference is a general review article discussing both stable and transient methods of gene therapy. Most importantly, the two cited references, taken as a whole would not suggest the instant invention to one of ordinary skill in the art.

The rejection of claims 1-4, 9-12, 14, 18, 20-26 and 28-30 under 35 USC §103 over Petkau in view of Alton *et al.*, Jaffe *et al.* and Wu *et al.* admits that none of the references specify that a superoxide dismutase (SOD) gene can be delivered by *in vivo* gene transfer. Nonetheless, the examiner uses the general teaching in the Petkau reference that the SOD protein reverses radiation and/or chemotherapy-induced leucopenia to springboard into an unsubstantiated statement that "it would have been obvious . . . to use existing methods of gene transfer (like those of Alton, Jaffe and Wu) to express SOD in vivo . . ." (Office Action, sentence bridging pages 7-8).

This rejection is factually and legally flawed. In the first instance, the examiner is making a mistake of fact because the Petkau reference's disclosure of a protein does not provide a gene for gene therapy. As to the mistake of law, the examiner provides no evidence that suggests the desirability of combining the references in a proposed manner. Such a combination is not available to preclude patentability under 35 USC § 103.

The third obviousness rejection of claims 1, 4, 9-12, 15, 19-26 and 28-30 over Lohrer *et al.* and Matsubara *et al.*, in view

of Mulligan is similarly defective as a matter of fact and law. The Lohrer reference only discusses the protective effect of the matallothionein protein and provides no motivation to deliver the metallothionein gene to provide transient expression of metallothionein to neutralize or eliminate a toxic ionic species.

The fourth obviousness rejection of claims 1-3, 9-11 and 28-30 over HockenbeRry *et al.* in view of Mulligan is defective because it applies a October 22, 1993 publication (Hockenberry) that is not available as a reference against Dr. Greenberger's October 15, 1993 filing date. The unavailable Hockenberry reference does discuss transfection of the MnSOD cDNA but differs from the instant invention in its aim to stably express MnSOD in vitro. (Hockenberry *et al.*, page 243, right column and page 249 at "Construction of Plasmids").

For the foregoing reasons, no combination of teachings from the references cited by the examiner in support of the obviousness rejections would have suggested providing polynucleotides *in vivo* to transiently express a protein that interacts with a toxic species to provide a protection against that toxic species. Applicant submits that the prior art disclosures of both (a) the use of exogenous superoxide dismutase protein to reverse the leucopenia induced by radiation and/or chemotherapy (Petkau) and (b) the induction of the endogenous metallothionein gene to provide resistance to alkylating agents and scavenge heavy metals (Mulligan) would not have suggested a gene therapy approach to achieve a protective effect in a therapeutic context (radiotherapy or chemotherapy).

Accordingly, the examiner has not established a *prima facie* case of obviousness. Since the enablement rejection is resolved by the enclosed declaration and remarks, it is believed that the application is now in condition for allowance. An early and favorable disposition of the application is earnestly solicited.

If the examiner for any reason feels that further discussion will assist him in examining the application, please call the undersigned attorney.

Respectfully submitted,

September 6, 1995
Date

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II. OBVIOUSNESS

Claims 1-4, 9-12, 14-15 and 18-30 are rejected under 35 USC §103. It seems that the examiner has found claims 5-8, 13, 16 and 17 to be novel and non-obvious in view of the prior art.

A. Three Remaining Obviousness Rejections

There are three remaining obviousness rejections: (1) Claims 1, 4 and 20-27 are rejected under 35 USC §103 as being unpatentable over Sorrentino *et al.* in view of Mulligan; (2) Claims 1-4, 9-12, 14, 18, 20-26 and 28-30 are rejected under 35 USC §103 as being unpatentable over Petkau in view of Alton *et al.*, Jaffe *et al.* and Wu *et al.* and (3) Claims 1, 4, 9-12, 15, 19-26 and 28-30 are rejected under 35 USC §103 as being

unpatentable over Lohrer *et al.* and Matsubara *et al.* in view of Mulligan as applied above.

Applicant maintains his argument that each of the three rejections are improper because the examiner has not shown that the prior art as a whole suggests the desirability to selectively combine the references he has chosen. The examiner presents no line of reasoning as to why the artisan reviewing only the collective teachings of the references would have found it obvious to selectively pick and choose various elements from the several references relied on to arrive at the claimed invention. The examiner has done little more than cite references to show that one or more elements or some combinations thereof is known.

The claimed invention is not directed to one or more new elements. Rather, Dr. Greenberger has invented a new method employing a new combination of elements. In fact, the references cited by the Examiner as disclosing particular elements of the invention do not disclose the elements of the claimed invention as well as the prior art cited within the instant specification and provided in the Information Disclosure Statement. It is impermissible for the examiner to use the claims as a frame and the prior art references as a mosaic to piece together a facsimile of the claimed invention.

B. First Obviousness Rejection Over Sorrentino in View of Mulligan

The first obviousness rejection of claims 1, 4, and 20-27 over Sorrentino in view of Mulligan admits that the Sorrentino reference is directed to *ex vivo* gene transfer, rather than *in vivo* gene therapy. However, the method of Sorrentino differs from the instant invention in several other respects: (1) retroviral vectors are used, (2) stable expression is desired and achieved and (3) the encoded protein does not neutralize or eliminate the toxic ionic species, but rather, extrudes it. The

Mulligan reference is a general review article discussing both stable and transient methods of gene therapy. Most importantly, the two cited references, taken as a whole would not suggest the instant invention to one of ordinary skill in the art.

1. The prior art provides neither a specific motivation to produce the invention as **claimed** nor evidence that the claimed method, which includes the combination of particular polynucleotides and transient expression, would be expected to characterize a successful method

The examiner alleges that "the objective of the claimed invention is the same as that in the Sorrentino reference - protection of a subject from chemotherapeutic toxicity - and the Mulligan reference provides the motivation to use alternate vector systems known in the art for gene therapy purposes" (Office Action at page 8, lines 9-14). The examiner does not allege that either reference provides the motivation to make the ***claimed*** invention. Only the general objective of the claimed invention, which is to protect a subject from chemotherapeutic toxicity, is contained within the Sorrentino reference. The general objective of Sorrentino is achieved using a method that differs from the instant invention in several other respects: (1) retroviral vectors are used, (2) stable expression is desired and achieved and (3) the encoded protein does not neutralize or eliminate the toxic ionic species, but rather, extrudes it. This rejection is flawed as a matter of law, as neither reference provides a specific motivation to produce the claimed invention. This rejection is flawed, also, as it relies on facts in Sorrentino that do not provide the particular guidance required by the specificity of the claims. Neither reference provides evidence that the claimed method would be expected to characterize a successful method.

Applicants traverse this reason for rejection because (1) there is no evidence of a motivation to have produced the

invention as *claimed* and (2) ther was no expectation that the claimed combination of polynucleotides and transient expression would provide a successful method. Where claimed subject matter has been rejected as obvious in view of a combination of prior-art references, a proper analysis under 35 USC §103 requires consideration of whether the prior art would have suggested using the *claimed* method, in light of contemporaneous methods, and whether that art also evidences a reasonable basis for one of ordinary skill to have expected success in modifying the prior-art methods of gene therapy. Both the suggestion and the reasonable expectation of success must be founded in the prior art, not in the applicant's disclosure. *In re Vaeck*, 20 USPQ2d 1438, 1442 (Fed. Cir. 1991).

2. The prior-art methods for administering a polynucleotide to protect a subject against toxic species would not have suggested using the claimed transient method because the skilled artisan would have expected the limitations associated with transient expression to be inadequate to produce an effective method

At the time that the instant patent application was filed, the specific combination of transient expression and the particular polynucleotides used for such expression was not suggested by the prior art. Accordingly, there could have been no reasonable expectation that a method for transient expression of protective genes would be capable of protecting a subject against a free radical, a superoxide anion or a heavy metal cation when the subject is exposed to ionizing radiation, clinical radiation therapy or a chemotherapeutic drug. Before the filing of the present application, therefore, the expectations of those in field militated against a method that would transiently express a protective gene product to protect a subject.

It is apparent, therefore, that no combination of teaching from the publication on a method to stably encode a protein that

extrudes a toxic ionic species and does not neutralize or eliminate the toxic ionic species (Sorrentino *et al.*) would have supported a reasonable expectation that stable transfection methods could be modified to achieve the claimed invention. For this reason the claimed invention is not obvious, within the meaning of §103, over the combination of (1) Sorrentino *et al.* in view of Mulligan.

C. Second Obviousness Rejection Over Petkau in View of Alton et al., Jaffe et al. and Wu et al.

The rejection of claims 1-4, 9-12, 14, 18, 20-26 and 28-30 under 35 USC §103 over Petkau in view of Alton *et al.*, Jaffe *et al.* and Wu *et al.* admits that none of the references specify that a superoxide dismutase (SOD) gene can be delivered by *in vivo* gene transfer. Nonetheless, the examiner uses the general teaching in the Petkau reference that the SOD protein reverses radiation and/or chemotherapy-induced leucopenia to springboard into an unsubstantiated statement that "but the secondary references do disclose in vivo adenoviral, liposomal, and DNA-conjugate gene transfer methods . . . which could be obviously employed to express SOD in vivo . . ." (Office Action, sentence bridging pages 8-9).

This rejection is factually and legally flawed. In the first instance, the examiner makes a mistake of fact because the Petkau reference, disclosing a protein, does not provide a gene for gene therapy. As to the mistake of law, the examiner provides no evidence that suggests the desirability of combining the references in a proposed manner. Such a combination is not available to preclude patentability under 35 USC § 103.

D. Third Obviousness Rejection Over Lohrer et al. and Matsubara et al., in view of Mulligan

The third obviousness rejection of claims 1, 4, 9-12, 15, 19-26 and 28-30 over Lohrer *et al.* and Matsubara *et al.*, in view of Mulligan is similarly defective as a matter of fact and law. The Lohrer reference only discusses the protective effect of the matallothionein protein and provides no motivation to deliver the metallothionein gene to provide transient expression of metallothionein to neutralize or eliminate a toxic ionic species.

None of the combined references in any of the obviousness rejections address the specific object of the instant invention to provide transient expression of the identified polynucleotides *in vivo*. For these reasons, the claimed invention is not obvious, within the meaning of §103, over the combination of (1) Sorrentino *et al.* in view of Mulligan; (2) Petkau in view of Alton *et al.*, Jaffe *et al.* and Wu *et al.* or (3) Lohrer *et al.* and Matsubara *et al.* in view of Mulligan *et al.*.

In view of the foregoing remarks and the submitted declaration, it is believed that the application is now in condition for allowance. A speedy and favorable disposition of the application is earnestly solicited.

Respectfully submitted,

1 April 1996
Date

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